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EFFECT OF VARIABLE VALENCE CATIONS ON THE BIOCIDAL PROPERTIES OF VITREOUS ENAMEL COATINGS

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It is shown that variable valence metal cations are promising for obtaining biocidal vitreous enamel coatings for household use. The biocidal action of vitreous coatings against the bacteria Escherichia coli and Pseudomonas auroginosa and the fungi Candida albicans and Aspergillus niger is determined by the presence in them of pigments containing cadmium selenides and sulfides. Fungicidal activity manifests for vitreous enamel coatings colored by pigments containing zinc and cobalt bichromates.

Key words: biocidal properties, variable valence cations, vitreous glass coatings.

The problem of increasing the biocidal action and biostability of household objects, buildings and structures is extremely important, since it has been found that microorganisms do many billions of dollars of damage worldwide. In addition, biologically contamination of living quarters cause infections in people and animals and engender epidemics [1].

Among pathogenic microorganisms that can invade organic and inorganic objects, aside from the widely occurring bacteria Escherichia coli (E. coli) and Presudomonas auroginosa (P. auroginosa), cord-forming fungi hold a dominant position. The damage done by fungi to different structures depends on the aggressive metabolic products, first and foremost, organic acids, that they produce. Cord-forming fungi are capable of growing rapidly and have powerful enzyme systems producing organic acids, which is what enables them to invade and corrode a large number of materials. Thus, the mold fungi belonging to the genus Aspergillus (black mold) are characterized by high biochemical activity. The yeast fungi of genus Candida are also pathogenic in live organisms; as saprophytes, these fungi live on the skin and mucous membranes of healthy people and are often responsible for micoses [1].

A large number of chemical compounds have been proposed to prevent sickness in people and animals and bio-corrosion of buildings and structures. These compounds are used as biocidal and anti-corrosion agents by introducing them into different materials as well as by treating the surfaces of buildings [2].

The chemical compounds can be divided into two groups on the basis of the mechanism by which the microorganisms act on cells. The first group comprises a large number of organic compounds, whose biocidal properties are due to the damage done to cell walls (lysozyme) or cytoplasmic membranes of microorganisms (phenols, chloroforms, cresols, neutral soaps, surfactants or detergents, esters, hydrogen ions, alcohols and toluenes) [1]. A review of the domestic and foreign scientific and technical literature and patents has shown that preparations containing guanidine are effective antimicrobial polymer preparations, used in the silicate industry [3]. Thus, the authors of [2] developed the preparation teflex A based on guanidine, which imparts fungicidal properties to silicate building materials.

The second group of chemical substances exhibiting microbicidal action on microorganisms that damage enzymes and interfere with exchange of substances are inorganic substances, such as carbon monoxide and certain active oxidizers — potassium permanganate, hydrogen peroxide as well as heavy-metal ions, which can interact with hydroxyl, sulfhydryl and carboxyl as well as amino groups, which can change the properties of proteins and coenzymes. Depending on the effectiveness of their action heavy-metal ions can be arranged in the following order: $Ag^{+} > Hg^{+2} > Cd^{2+} > Cu^{2+} > Au^{3+} > Ni^{2+} > Zn^{2+}$ [4].

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TABLE 1. Optical Density and Initial Concentration of Bioassay Cultures

Indicator	E. coli	P. auroginosa	C. albicans	A. niger
D	0.03	0.035	0.035	0.4
C_{ini}, cells/cm^3	1.1×10^3	1.8×10^5	1.9×10^4	1.1×10^6

One method of protection from pathogenic bacteria and fungi is to develop and adopt competitive, universal, protective-decorative silicate materials, including glass coatings with biocidal action for steelware for medical, pharmaceutical and household use [5].

Inorganic nanopowders based on silver [6], hydroxyapatite modified with titanium oxide [7], zinc and copper phosphates and oxides [8, 9] as well as zinc titanates [10] are widely used to impart biocidal action to vitreous enamel coatings. In order to maintain admissible migration for toxic substances variable valence metal ions as biocidal agents can be introduced in the form of oxides or salts into batch used for glass materials as well as into slip. Thus, the following oxides were introduced into glass compositions in order to fight against mold fungi (wt.%): 0.25 Ag_2O, 2 CuO, to 8 CdO, 0.9 As_2O_3, 3 TiO_2, to 17 ZnO or 8.5 PbO [11]. It is established in [12] that the introduction of antimicrobial agents based on Ag^+, Zn^{2+} and Cu^{2+} into slip in amounts from 1 to 10 parts per 100 parts frit in the case of glass coatings for plumbing articles eliminates 70.7% of the staphylococcus bacteria.

Today variable valence metal ions are widely used to color glass and enamel. However, the use of these oligodynamic components in enameled dishware and household appliances which come into contact with food products and water can be dangerous to humans and must be tightly regulated [13, 14]. The coloring process is effectuated by introducing metal ions during melting and into slip as pigments [13] or salts [15].

The intense development of the enameling industry requires perfecting and adopting new types of competitive protective and decorative coatings for household use. Colored vitreous enamels with biocidal functions with respect to a wide spectrum of pathogenic microorganisms are of greatest interest. The objective of the present work was to obtain biocidal vitreous enamel coatings for household use colored by cationic variable-valence metals.

EXPERIMENTAL PROCEDURE

The fungicidal and bactericidal properties of vitreous coatings were determined using solid and liquid media by diffusion [16] and quantitative [17] methods according to the following scheme: selection of cultures of microorganisms for bioassays → preparation of inoculum → standardization of bioassay inoculum → study of biocidal properties of test samples by diffusion and quantitative methods.

TABLE 2. Scale for Evaluating the Biocidal Action of Materials [16]

Diameter of microorganism growth suppression zone (L, mm)	Degree of bactericidal action of building materials on a bioassay microbe
Absence of microbe growth suppression zone around disks	No bactericidal action
Microbe growth suppression zone from 10 to 15 mm	Weak bactericidal action
Same from 15 to 20 mm	Moderate bactericidal action
Same, > 20 mm	Strong bactericidal action

The following laboratory cultures of opportunistic bacteria and fungi were used for bioassays:

– bacteria belonging to a group of rod-shaped colonic bacteria E. coli and rod-shaped pyocyanin bacteria P. aeruginosa;

– mold fungi Aspergillus niger (A. niger); yeast fungi Candida albicans (C. albicans).

The biomass of the bacteria was cultured on agar slants with a selective 2% beef peptone agar (BPA) and beef peptone bullion (BPB) at temperature 35 ± 2°C; the biomass of the fungi was cultured on Chapek–Dox selective agar slants at temperature 26 ± 2°C. Ten milliliters of sterile bullion were inoculated with a definite volume of a daily culture of each type of bioassay medium.

The bioassay inoculum was standardized by determining the total number of microbes and the optical density. In the first case a definite volume of inoculum of the bioassay culture was taken from a test tube and a series of cultures was prepared for seeding on a dense BPA medium. The optical density D of bioassays for each corresponding culture was determined on FÉK-M photocolorimeter by a colorimetric method. The density of the cells in the control samples was determined from plots of the optical density of the medium versus the corresponding concentration of bacteria C_{ini} cells/cm^3 (Table 1).

The control samples were comprised of the following:

– test tubes with a medium with no bioassays but with the experimental samples (K2);

– test tubes containing one medium BPB (control for sterility of the medium) (K3).

The test samples consisted of bilaterally enameled, 1.5 cm in diameter, steel disks.

Using the diffusion method, the biocidal properties of test samples were checked with respect to microorganisms, grown on dense nutrient media, by visual evaluation of their growth. In the case suppression of bioassay growth was detected the diameter of the microbe growth suppression zone was measured (L, mm) around the test sample disk, taking account of its diameter (Table 2).

The bioassay microorganisms were seeded with the inoculum by the lawn method. Ten to twenty milliliters of the melted and cooled to 40°C BPA medium or Chapek–Dox

agar medium were placed into a Petri dish, the medium was allowed to solidify and then 0.5 ml of inoculum was introduced onto the surface of the solidified plate. Using a spatula, the inoculum was spread over the surface and allowed to dry for 0.5 h. Next, disks of assay samples of the experimental variants, pre-moistened with distilled water, were placed on the seeded agar plate. The dishes with the disks were kept at room temperature for 3 h and then placed upside down in a thermostat. The dishes seeded with bacteria were incubated for 24 h at temperature 35 ± 2°C. The dishes seeded with microscopic fungi were incubated for 7 days at temperature 26 ± 2°C.

The quantitative, or counting, method is based on checking the growth level of the bioassay microorganisms inoculating the liquid nutrient media in the presence of and without bioassay samples. To determine the biocidal properties of the bioassay samples 0.1 ml of inoculum of the corresponding bioassay and bioassay sample were introduced into the experimental test tube with 6 ml of the BPA medium. From each experimental test tube with the medium, inoculated with the corresponding bioassay, 0.1 ml of inoculum were extracted and seeded on a BPA medium. For the bioassay inoculum a BPA medium, divided beforehand by a factor of 100, was seeded with 0.1 ml. The optical density of the initial inoculum of each bioassay was determined with a FÉK-M photocolorimeter at $\lambda = 490$ nm and sensitivity equal to 3. The initial concentration C_{ini} for each bioassay is presented in Table 1. To determine the biocidal action of bioassay samples all test tubes with the control and experimental samples were incubated in a thermostat at temperature 37°C for 24 h. After incubation of the samples the optical density was remeasured for test tubes with control and experimental samples and by comparing with the standard solution.

EXPERIMENTAL PART

The presence of variable valence metal cations in pigments is a necessary condition to guarantee a biocidal effect in the experimental vitreous enamel coatings.

X-ray phase analysis (XPA) was performed on the pigments to determine their phase composition. XPA showed that the blue pigment is characterized by the presence of Co_2O_4, yellow pigment by $K_2Cr_2O_7$, green pigment by CrO, Cr_2O_3, $ZnCr_2O_4$ and $CoCr_2O_4$, claret pigment by $CdS_{0.42}Se_{0.58}$, $CdS_{0.52}Se_{0.48}$, orange pigment by $CdS_{0.54}Se_{0.46}$, $Cd_{10}S_{8.13}Se_{1.87}$, $Cd_{10}S_{5.71}Se_{4.2}$ and emerald pigment by Co_2O_4, $ZnCrO_4$.

The elemental mass content in pigments was determined by energy dispersive x-ray fluorescence analysis with a SPRUT spectrometer. It was determined that the pigments are characterized by the presence of the following elements: blue — 100% Co, yellow — 42.91% K, 57.02% Cr, green — 6.068% Ti, 53.321% Cr, 0.536% Mn, 20.466% Co, 1.146% Ni, 18.256% Zn, 0.204% Zr, claret — 0.048% Cu, 0.415% Zn, 25.005% Se, 74.532% Cd, orange — 10.613% Se, 89.387% Cd, emerald — 48.86% Cr, 17.784% Co, 24.39% Zn, 8.967% Zr.

Pigments containing variable valence metal cations were chosen as oligodynamic components to obtain colored biocidal vitreous enamel coatings for enameling the exterior parts of cookware. The yellow, claret and orange pigments were introduced in the amount (by weight) 1 part per 100 parts slip; blue, green and emerald pigments were introduced in the amount 4 parts per 100 parts slip while grinding a slip composition consisting of titanium vitreous enamel frit ÉSP-143 — 100.0 parts, clay — 6.0 parts, potassium chloride — 0.05 parts, and water — 50.0 parts. Slips were deposited on 0.7 mm thick samples of 06kp low-carbon steel after which the coatings were dried and fired at temperature 820 – 840°C.

It should be noted that pigments containing cadmium and selenium can be used only to enamel the exterior of housewares that do not come into contact with food products and water. These metals are class-II hazards, are highly toxic, engender dermatitis and burns and are characterized by cumulative action [18]. The toxic concentration for cadmium is 150 mg/kg. Selenium is fatal to humans at 2 – 4 mg/kg [18]. According to [19] the maximum admissible concentration is 0.001 mg/ml for Cd and 0.01 mg/ml for Se. The admissible migration concentration on contact of food products with enameled housewares is 0.1 mg/ml for Cr, Co and Cu and 1.0 mg/ml for Zn [20].

According to REACH Directive 67/546/EEC CdSe is a harmful substance (Xn) with R (risk) phrases R21, R23/25, R33 and R50/53; S-phrases S36/37, S45, S60, S61. CdS is carcinogenic *T* with R45, R22, R48/23/25, R62, R63, R68, R50/53; S53, S45, S61 the concentration *C* lies in the range $1.0\% \le C \le 5\%$, *T*: R 45-48/20-22/68, $0.1\% \le C \le 1\%$, *T*: R 45-48/20-22.

The biocidal properties were determined for a pigment-free vitreous enamel coating (test-sample 1) and for vitreous enamel coatings with blue pigment (test-sample 2), yellow pigment (test-sample 3), green pigment (test-sample 4), claret pigment (test-sample 5), orange pigment (test-sample 6) and emerald pigment (test-sample 7).

RESULTS AND DISCUSSION

The results of the determination of the biocidal properties by the diffusion method on the dense nutrient medium BPA for test-samples 1 – 3 showed no growth suppression for all experimental bioassay cultures.

After 24 h it was observed that against the background of the “continuous lawn” biomass zones with lower growth density were observed around test-samples 4 – 7 for the bacteria Escherichia coli and Pseudomonas aurogenosa (see Fig. 1). Thus, a change in the growth zone of colonies for Escherichia coli around test-samples 6 and 7 is greater than 20 mm and is characterized as strong bactericidal action; for

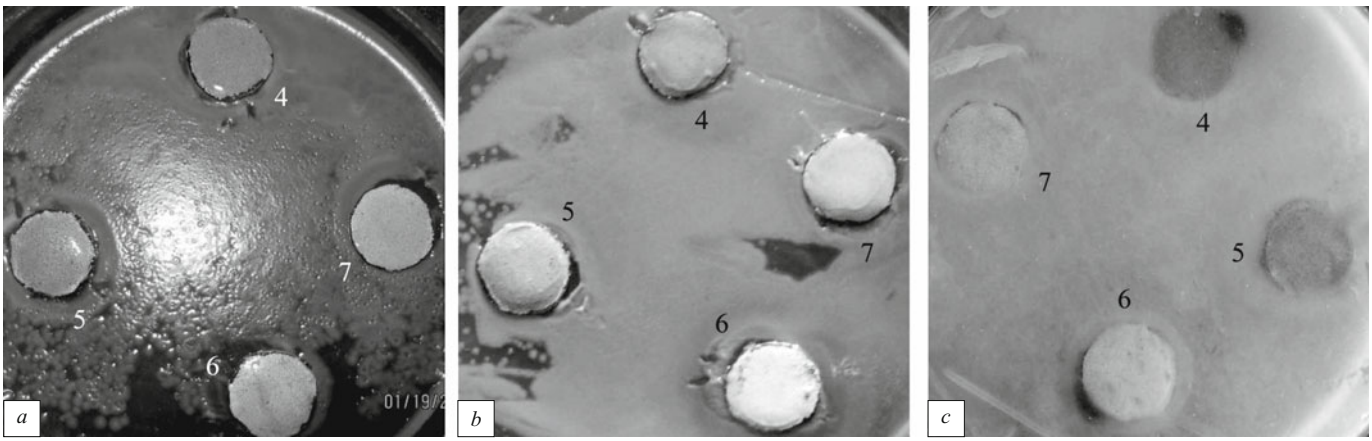

Fig. 1. Growth suppression zone for bioassays on BPA for contact with test-samples: *a*) Escherichia coli; *b*) Pseudomonas auroginosa; *c*) Candida albicans.

test-samples 4 and 5 with moderate bactericidal action the growth suppression zone is about 20 mm [16] (see Fig. l). For the Pseudomonas aurogenosa culture a zone of moderate bactericidal action is observed only for test-sample 6. Zones of dense growth of biomass around test-samples 4 – 7 are observed for yeast fungi Candida albicans (see Fig. 1), which is an indication of intense growth of microorganisms. Likewise, no fungicidal effect with respect to mold fungi Aspergillus niger was observed for test-samples 4 – 7.

The determination of the antibactericidal and fungicidal properties by the diffusion method is revealing and can be used for primary determination of biocidal action. But, since this method, which is used for migrating compounds, does not give a complete picture, it is necessary to study in parallel the biocidal properties in a liquid medium, which is more reactive with respect to vitreous enamels.

Checks for sterility of the medium (K3) and for a medium without bioassays with the samples being studied (K2) were made. They showed absence of bacteria in these control samples, which is necessary for a clean experiment. The effectiveness of the test-samples in a bioassay medium was evaluated according to its growth indicator in comparison with the medium inoculated only with the bioassay without adding the experimental samples (K1).

The results showed that the concentration of Escherichia coli cells after 24 h of growth for test-samples 5 and 6 decreased approximately 10-fold compared with the control K1. For test-samples 1 – 4 and 7 the concentration of the cells of this bioassay is comparable to the control sample K1 (Table 3). The Pseudomonas aurogenosa cell concentrations in the experimental test-samples 3, 4 and 7 and in the control sample are approximately the same, and for test-samples 5 and 6 the concentration decreased very little (see Table 3). For test-samples 1 – 3 a very small increase of growth of microorganisms, as compared with the control sample, is observed on contact with Pseudomonas aurogenosa. For the

TABLE 3. Variation of the Optical Density and Concentration of the Bioassays for Interaction with Test-Samples by the Quantitative Method

Test samples and controls	Bioassay							
	Escherichia coli		Pseudomonas auroginosa		Candida albicans		Aspergillus niger	
	D	*C*, cells/cm^3	*D*	*C*, cells/cm^3	*D*	*C*, cells/cm^3	*D*	*C*, cells/cm^3
1	0.22	6.5×10^4	0.70	2.0×10^6	0.23	1.7×10^5	0.30	1.40×10^6
2	0.20	5.5×10^4	0.70	2.0×10^6	0.28	2.0×10^5	0.34	1.55×10^6
3	0.24	6.6×10^4	0.59	1.7×10^6	0.25	1.85×10^5	0.45	1.98×10^6
4	0.21	5.6×10^4	0.60	1.80×10^6	0.15	1.12×10^5	0.05	2.1×10^5
5	0.05	6.0×10^3	0.40	1.26×10^6	0.06	3.8×10^4	0.035	1.4×10^5
6	0.045	5.8×10^3	0.50	1.32×10^6	0.05	2.8×10^4	0.022	0.9×10^5
7	0.18	4.4×10^4	0.59	1.78×10^6	0.14	1.0×10^5	0.10	4.2×10^5
K1	0.24	6.7×10^4	0.60	1.80×10^6	0.34	2.4×10^5	0.26	1.1×10^6
K2	0	0	0	0	0	0	0	0
K3	0	0	0	0	0	0	0	0

TABLE 4. Comparative Assessment of the Biocidal Properties of Test-Samples after Interaction with Bioassay Cultures

Test samples	Phase composition of pigment	Method of determining biocidal activity*							
		Diffusion method				Quantitative method			
		Bioassay							
		E. coli	P. auroginosa	C. albicans	A. niger	E. coli	P. auroginosa	C. albicans	A. niger
1	No pigment	–	–	–	–	–	–	–	–
2	Co_2O_4,	–	–	–	–	–	–	–	–
3	$K_2Cr_2O_7$	–	–	–	–	–	–	–	–
4	$ZnCr_2O_4CoCr_2O_4$, Cr_2O_3	+	–	–	–	–	–	+	+
5	CdS_xSe_y	+	–	–	–	+	+	+	+
6	CdS_xSe_y	+	+	–	–	+	+	+	+
7	Co_2O_4, $ZnCrO_4$	+	–	–	–	–	–	+	+

* (+) — presence of biocidal activity; (–) — absence of biocidal activity.

cells of the bioassay Candida albicans the concentration is lower than in the control sample by a factor of approximately 2 in the experiments with test-samples 4 and 7, a factor of 6 for test-sample 5, and a factor of 9 for test-sample 6. For cells of the bioassay Aspergillus niger the concentration is 2.6 times lower in experiments with test-sample 7 than in the control sample, 5.2 times lower for test-sample 4 and 7.8 times lower for test-sample 6. The test-samples 1 – 4 did not show biocidal action with respect to yeast and mold fungi (see Table 3).

The study of the biocidal properties by the diffusion method on solid media showed that the test-samples 4 – 7 are characterized by an antibactericidal effect with respect to Escherichia coli. An antibactericial effect with respect to Pseudomonas auroginosa is observed only for test-sample 6 (see Table 3).

Evaluation of the biocidal effect by the quantitative method in liquid media showed that the test-samples 5 and 6 exhibited the strongest inhibitory action with respect to all bioassays studied. The test-samples 4 and 7 also exhibited fungicidal action with respect to Candida albicans and Aspergillus niger. The presence or absence of biocidal activity for vitreous enamel coatings with different pigments is indicated in Table 4.

CONCLUSIONS

The biocidal action of vitreous enamel coatings with respect to the bacteria Escherichia coli, Pseudomonas auroginosa and the fungi Candida albicans, Aspergillus niger is determined by the presence in them of pigments containing CdS_xSe_y. The functional activity is manifested for vitreous glass coatings colored by pigments containing $ZnCr_2O_4$ and $CoCr_2O_4$.

The biocidal vitreous glass coatings studied here can be used to protect assorted enameled steelware depending on its inhibitory action.

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